Non-anastomotic biliary strictures after liver transplantation
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Changes in cholangiocyte bile salt transporter expression after mouse orthotopic liver transplantation and the role of bile salts

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ABSTRACT

Background Bile salts cycle between cholangiocytes and hepatocytes through a cholehepatic shunt pathway. The role of bile salt absorption and excretion through cholangiocytes in the development of bile duct injury after orthotopic liver transplantation (OLT) is unknown. We therefore analyzed changes in cholangiocyte bile salt transporter expression in relation to bile composition and biliary injury in a mouse model of OLT. Methods Livers from wild-type mice or mice heterozygous for disruption of the multidrug resistance 2 gene (Mdr2<sup>+/−</sup>) were transplanted into wild-type recipients. Mdr2<sup>+/−</sup> mice have normal liver histology and function under normal conditions, but secrete only 50% of the normal amount of phospholipids into their bile, leading to an abnormally high bile salt/ phospholipid ratio. This has been associated with excessive bile duct injury after OLT. Expression of cholangiocyte bile salt transporters Asbt, Ost-alpha/beta was assessed by western blotting and RT-PCR. Levels were correlated with the biliary bile salt/phospholipid ratio and hepatic expression of TNF-alpha and IL1-beta mRNA. Results At baseline, Asbt and Ost-beta protein expression were significantly increased in Mdr2<sup>+/−</sup> livers, compared to wild-type livers. Transplantation of wild-type livers did not result in significant changes in transporter expression. Transplantation of Mdr2<sup>+/−</sup> livers, however, resulted in down-regulation of mRNA expression for Asbt, Ost-beta and Ost-alpha after OLT. While levels of Asbt and Ost-alpha mRNA correlated significantly with the biliary bile salt/phospholipid ratio, there was a strong negative correlation between Ost-alpha/beta mRNA expression and the expression of TNF-alpha and IL1-beta. Conclusions Asbt expression is regulated in direct proportion to the biliary bile salt/phospholipid ratio. Unbalanced reduction of Asbt and Ost-alpha/beta expression after OLT may result in bile salt retention in cholangiocytes, which may result in cytotoxicity and aggravate bile duct injury.
INTRODUCTION

Insight in the role of bile salts in the pathogenesis of bile duct injury following orthotopic liver transplantation (OLT) has gradually emerged during the last decade. Bile salts have detergent properties towards cell membranes and intracellular accumulation may act toxic to cells. Cholangiocytes are constantly exposed to very high bile salt concentrations (1, 2). Normally, the toxic effects of bile salts are prevented by formation of complexes (mixed micelles) with phospholipids. An increased biliary bile salt/phospholipid ratio, e.g. due to reduced secretion of phospholipids, has been associated with increased bile salt-induced hepatobiliary injury. For example, mutations in the MDR3 gene, leading to a decreased expression of MDR3, the transporter of phospholipids into the bile, have been associated with a phenotype of bile duct injury and intrahepatic cholestasis (e.g. progressive familial intrahepatic cholestasis (PFIC) type 3 and intrahepatic cholestasis of pregnancy) (3, 4). Accumulating evidence indicates that bile salt toxicity is also involved in the pathogenesis of bile duct injury after OLT (5-8). In a mouse model of arterialized OLT, we have previously shown increased hepatobiliary injury after transplantation of livers from donors heterozygous for disruption of the Mdr2 gene (Mdr2+/−; the homologue of human MDR3), compared to wild-type donor livers (6). These heterozygous Mdr2+/− mice secrete approximately half of the normal amount of phospholipids into their bile, resulting in an abnormal high biliary bile salt/phospholipid ratio. In contrast to their homozygous littermates which completely lack biliary phospholipids, Mdr2+/− mice do not develop bile duct injury under normal conditions. However, when livers from Mdr2+/− mice were transplanted into wild-type recipients severe biliary injury developed, leading to cholestatic phenotype, periportal inflammation and ductular proliferation. These data indicated that bile salts aggravate hepatobiliary injury after cold ischemia and subsequent reperfusion as occurs in a liver transplant procedure. Also other experimental (7, 9) and clinical studies (5, 8) have provided evidence that bile salts are involved in the pathogenesis of biliary injury after OLT.

Bile composition and flow is not only determined by hepatocyte bile transporters, but also by transporters located in cholangiocytes. Cholangiocytes express various bile salt transporters such as the apical sodium-dependent bile salt transporter (Asbt) and the heteromeric organic solute transporter Ost (consisting of the two half transporters Ost-alpha and beta), responsible for the uptake and secretion of bile salts, respectively.
The re-absorption of bile salts from the bile by cholangiocytes and the subsequent secretion into the peribiliary arterial circulation, leading them back to the hepatocytes for renewed secretion, is also known as the cholehepatic shunt pathway of bile salts. To date, a comprehensive understanding of the role of cholangiocyte transporters in the pathogenesis of bile duct injury after OLT is lacking. Dysregulation of cholangiocyte bile transporters after OLT could theoretically contribute to the intracellular accumulation of toxic bile salt in cholangiocytes, thereby contributing to biliary injury and perpetuating a periportal inflammatory response as has recently been described by Wagner et al.

In the current study, we aimed to investigate whether OLT leads to changes in the expression of the cholangiocyte transporters involved in the cholehepatic shunt pathway. We therefore analyzed gene and protein expression of Asbt and Ostalpha/Ost-beta in our previously established mouse OLT model. To determine the impact of biliary bile salts and the bile salt/phospholipid ratio on the expression of these transporters, we compared livers transplanted from Mdr2+/+ mice with livers from wild-type donors. To determine whether changes in these cholangiocyte transporters are associated with an inflammatory response, we also assessed the hepatic expression for TNF-alpha and IL1-beta in the donor livers and correlated this with the expression levels of the cholangiocyte transporters.

**MATERIALS AND METHODS**

**Animals and the liver transplant model**

A murine model of arterialized OLT was used as described before. In summary: livers (n=5 for each group) from inbred male wild-type or Mdr2+/− mice (FVB.129P2-Abcb4tm1Bor, The Jackson Laboratories, Maine, USA), were transplanted into wild-type mice in an orthotopic fashion using both portal venous and with arterial reconstruction, as described earlier by Tian et al. After procurement, liver grafts were stored in cold (4°C) Ringer’s solution for 60 minutes until implantation in the recipient. Anhepatic time in the recipient was consistently kept below 20 minutes. Separate groups of wild-type and heterozygous mice (n=5 for each group) were obtained for determination of baseline values. Recipient animals were sacrificed 2 weeks after OLT. After laparotomy, the bile duct was canulated and bile was collected. Subsequently, blood was collected from the inferior vena cava and the liver was rapidly excised and processed for further analysis.
**Real-Time quantitative PCR**

Total RNA was isolated from frozen mouse liver using TRIzol reagent and reverse transcription was performed using random hexamer, according to the manufacturer’s instructions (Invitrogen life technologies, Basel, Switzerland). TaqMan gene expression assays (PE Applied Biosystems) for TNF-alpha (assay ID Mm00443258_m1), IL1-beta (assay ID Mm00434228_m1), Asbt (assay ID Mm00488258_m1), Ost-alpha (assay ID Mm00521531_m1) and Ost-beta (assay ID Mm00619242_m1) were used to quantify mRNA expression after normalization for expression of 18S ribosomal RNA, using the ABI Prism 7000 Sequence Detector (Applied Biosystems).

**Membrane isolation and western blotting**

Isolation and processing of membrane fractions for western blotting were performed as described before (6). Blots were probed with antibodies against Asbt, Ost-alpha and Ost-beta at appropriate dilutions (antibodies were kindly provided by Paul Dawson, Wake Forest University School of Medicine, USA) (14). In addition, blots were reprobed with an anti-beta-actin antibody (Abcam, Cambridge, UK) to confirm equal protein loading. Immune detection was assessed by using the ECL chemiluminescent detection system (Amersham, UK). For comparison of expression levels, autoradiographs were scanned with the CAMAG TLC scanner II (Camag AG, Muttenz, Switzerland).

**Determination of bile salts and phospholipids**

Total biliary bile salt concentrations were measured spectrophotometrically using 3α-hydroxysteroid dehydrogenase (15). Biliary phospholipid concentration was analyzed using a commercially available enzymatic method (Wako Chemicals GmbH, Neuss, Germany).

**Statistical analysis**

Values are expressed as mean ± SD. Data was analyzed using SPSS software version 16.0 for Windows. Differences within and between groups were compared using the 2-tailed independent-Samples T test. For correlation analyses the Pearson test was used. All P-values were considered as statistically significant at a level of less than 0.05.
RESULTS

Expression of Asbt and OSTalpha/beta in donor livers before and after OLT

Changes in Asbt, Ost-alpha and Ost-beta mRNA expression are presented in Figure 1. In wild-type mice, there were no major changes in the mRNA expression of Asbt and Ost-alpha at 14 days after transplantation, compared with pretransplant levels. Ost-beta mRNA levels were about 4-fold lower after transplantation, but this did not reach statistical significance due to the large variation in baseline expression levels. In Mdr2+/− livers, baseline mRNA levels for Asbt, Ost-alpha and Ost-Beta were higher than in wild-type livers, but this not reach statistical significance. After OLT, however, Asbt and Ost-beta mRNA levels were significantly decreased compared to pretransplant levels. In parallel with the stable mRNA expression levels in wild-type livers after transplantation, there were also no significant changes in Asbt, Ost-alpha and Ost-beta protein expression. In Mdr2+/− liver grafts, however, protein levels of Asbt and Ost-beta were significantly increased before transplantation, compared with levels in wild-type livers. After transplantation, protein expression of all three transporters in Mdr2+/− livers decreased significantly compared to pretransplant values (Figure 2).

Relationship between transporter expression and biliary bile salt/phospholipid ratio

To determine whether the expression of Asbt, Ost-alpha, or Ost-beta is possibly influenced by the biliary bile salt concentration, we next correlated mRNA levels for these transporters with the biliary bile salt/phospholipid ratio in wild-type and Mdr2+/− liver grafts. Asbt and Ost-alpha and mRNA correlated significantly with the biliary bile salt/phospholipid ratio ($R=0.832$ and $R=0.616$, respectively), whereas Ost-beta mRNA showed no relationship with the biliary bile salt/phospholipid ratio (Figure 3).
Relative transcript levels of Asbt, Ost-alpha and Ost-beta.

mRNA coding for Asbt, Ost-alpha and Ost-beta were quantified by RT-PCR (n=5 for each group) in wild-type (WT) and Mdr2+/− livers before (pre-OLT) and two weeks after OLT (post-OLT). The number of transcripts was normalized to the wild-type baseline levels. Data shown are mean +/- SD and presented in a semi-logarithmic way. Before versus after OLT, WT mRNA livers expression for Asbt, Ost-alpha and Ost-beta was not significantly different. Baseline Mdr2+/− mRNA expression for Asbt, Ost-alpha and Ost-beta showed a tendency to increase, however did not reach significance. Likewise protein regulation, expression for Asbt, Ost-beta was decreased significantly in Mdr2+/- livers, before versus after OLT. Compared to WT, none of the bile salt transporters Mdr2+/- livers after showed significant different expression; *P≤0.05 compared to WT at the same time point, †P≤0.05 compared to pre-OLT.
Figure 2
Relative protein expression for Asbt, Ost-alpha en Ost-beta.

(A) Western blot analysis of Asbt (top row), Ost-alpha (second row) and Ost-beta (bottom row) from liver extracts (n=3 for each group) in wild-type (WT) and Mdr2\textsuperscript{+-} liver grafts before (pre-OLT) and two weeks after OLT (post-OLT). Changes in Asbt, Ost-alpha en Ost-beta were standardized for beta-actin protein and quantified.

(B) Protein levels were normalized to the wild-type baseline levels. Data shown are mean +/- SD and presented in a semi-logarithmic way. No clear differences in expression for Asbt (48 kDa), Ost-alpha (40 kDa) and Ost-beta (24 kDa) were observed in wild-type mice before and after OLT. Compared to WT, Mdr2\textsuperscript{+-} expression for Asbt and Ost-beta before OLT was significantly increased. Before versus after OLT, all bile salt transporters of Mdr2\textsuperscript{+-} livers were down-regulated significantly. Compared to pre-OLT, Mdr2\textsuperscript{+-} Ost-alpha and Ost-beta expression after OLT was significantly decreased; \textsuperscript{a}P<0.05 compared to WT at the same time point, \textsuperscript{b}P<0.05 compared to pre-OLT.
Figure 3

A

Fold induction A sbt

biliary bile salt / phospholipid ratio

$\text{r} = 0.832$

$p < 0.001$

B

Fold induction Ost-alpha

biliary bile salt / phospholipid ratio

$\text{r} = 0.618$

$p = 0.004$
Correlation between Asbt and OST-alpha/beta, and the biliary bile salt/phospholipid ratio.

(A) Asbt mRNA expression correlated strongly with the bile/phospholipid ratio ($R=0.832$, $P<0.001$), whereas (B) Ost-alpha mRNA expression correlated slightly with the bile salt/phospholipid ratio ($R=0.616$, $P=0.004$). (C) mRNA expression for Ost-beta did not correlate with the biliary bile salt/phospholipid ratio.

Relationship between bile transporter and pro-inflammatory cytokine expression

To study whether changes in the expression of the bile transporters were associated with changes in the expression of the pro-inflammatory cytokines, we determined mRNA levels for TNF-alpha and IL1-beta in wild-type livers and Mdr2$^{-/-}$ livers. In addition, we correlated Asbt and Ost-alpha/beta mRNA levels with the expression levels of these pro-inflammatory cytokines. TNF-alpha and IL1-beta mRNA expression levels were significantly increased after transplantation, especially in Mdr2$^{-/-}$ livers (Figure 4). There was a strong negative correlation between expression of Ost-alpha and Ost-beta and the expression of TNF-alpha ($R=0.504$ and $R=0.687$, respectively). In addition, there was a strong negative correlation between Ost-beta expression and IL1-beta expression ($R=-0.554$) (Figure 5).
Figure 4
Relative transcript levels of TNF-alpha and IL1-beta.

mRNA coding for TNF-alpha and IL1-beta were quantified by RT-PCR (n=5 for each group) in wild-type (WT) and Mdr2+/- livers before (pre-OLT) and two weeks after OLT (post-OLT). The number of transcripts was normalized to wild-type baseline levels. Data shown are mean +/- SD and presented in a semi-logarithmic way. In wild-type livers after OLT, mRNA expression for both TNF-alpha and IL1-beta was increased significantly. There were no differences in baseline TNF-alpha and IL1-beta mRNA expression between Mdr2+/- and wild-type mice. mRNA expression for TNF-alpha and IL1-beta in Mdr2+/- livers was significantly increased after OLT, compared to both baseline Mdr2+/- and wild-type livers mRNA levels after OLT; ^P≤0.05 compared to WT at the same time point, ^P≤0.05 compared to pre-OLT.
Figure 5
Correlation between Asbt and OST-alpha/beta, and TNF-alpha and IL1-beta.
(A) Ost-alpha mRNA expression correlated significantly negative with the bile/phospholipid ratio ($R=-0.504, P=0.023$), whereas (B) Ost-alpha mRNA expression did not correlate with IL1-beta.
(C-D) Ost-beta mRNA expression correlated significantly negative with the TNF-alpha ($R=-0.687, P=0.001$) and IL1-beta ($R=-0.554, P=0.011$).
DISCUSSION

The goal of this study was to determine whether OLT is associated with changes in the expression of the cholangiocyte bile salt transporters Asbt and Ost-alpha/beta and whether expression levels of these transporters correlate with biliary bile salt/phospholipid ratio or with the expression of pro-inflammatory cytokines. The cholangiocyte bile salt transporters are involved in the cholehepatic shunt pathway, characterized by the reabsorption of bile salts from the bile and the subsequent secretion into the peribiliary capillary plexus, leading them back to the hepatocytes. Disruption of this process may result in the accumulation of toxic bile salts in cholangiocytes contributing to cellular injury after OLT.

Although Mdr2+/- mice display normal histology and function of the liver under physiological conditions, we observed an increased expression of Asbt and Ost-beta protein at baseline in Mdr2+/- livers, compared to wild-type livers. This strongly suggests an increased activation of the cholehepatic shunt in Mdr2+/- mice, which likely results from the relatively high biliary bile salt/phospholipid ratio in these mice. This is in line with the observed strong correlation between Asbt (and to some extend Ost-alpha/beta) mRNA expression and the biliary bile salt / phospholipid ratio after transplantation. In our mouse OLT model, we observed a discrepancy between mRNA and protein expression levels of Asbt and Ost-beta, suggesting modifications at the posttranscriptional level (16).

A relationship between Asbt expression and bile salts has previously been described in rat cholangiocytes (17-19). Asbt expression in rat cholangiocytes is chronically regulated in direct proportion to biliary bile salt concentration, thereby maintaining the biliary bile salt concentration (17-19). Depletion of biliary bile salts by external biliary drainage has shown to result in a marked decrease in cholangiocyte Asbt gene and protein expression and reduced transporter activity (20). Infusion of taurocholate in bile salt-depleted rats results in a restoration of Asbt gene and protein expression and transport activity. Our observations are in line with a bile salt controlled expression of Asbt in cholangiocytes.

Ost-alpha and beta are considered to be important transporters at the basolateral membrane of cholangiocytes. Secretion of bile salts through Ost-alpha/-beta is required to avoid potentially toxic accumulation of intracellular bile salts (21, 22). Expression of the Ost-beta subunit is reported to be regulated by the farnesoid X receptor and
was shown to be more sensitive to bile salts than the Ost-alpha subunit (21, 23). Our data suggest that after OLT the expression of OST-alpha/beta is not only influenced by biliary bile salts, but may also be affected by pro-inflammatory cytokines such as TNF-alpha and IL1-beta. The strong negative correlation between this transporter and the expression of TNF-alpha and IL1-beta suggests that the pro-inflammatory cytokines may result in a reduced expression of Ost-alpha/beta as well. Altogether, this results in a differential expression of the uptake transporter Asbt and the secretory transporter Ost-alpha/beta, causing a dysbalance in bile salt reabsorption and secretion by cholangiocytes. This may subsequently result in accumulation of bile salts in cholangiocytes, causing cell damage and aggravation of bile duct injury.

This study provides new insight into the mechanisms underlying bile duct epithelial damage after liver transplantation. Excessive bile duct damage during and after transplantation may result in narrowing of bile ducts, also known as non-anastomotic strictures (NAS) or ischemic-type biliary lesions (ITBL) (24). Several investigators have tried to unravel the pathogenesis of NAS. It has become clear that NAS is most likely a type of biliary complication that has various different causes. Both ischemic injury, immune-mediated injury, and bile salt-mediated injury of the biliary epithelium are considered to be involved in the development of NAS. In a clinical study, we recently reported an association between altered bile composition, characterized by a high bile salt/phospholipid ratio, and the development of NAS after transplantation (8). The mechanisms by which bile salts play a role in the development of bile duct injury remain to be determined. It is well known that bile salts have potent detergent properties and this may cause disruption of cellular membranes. However, bile salts may also have a more intracellular toxic effect (16). The current study suggest that an unbalanced expression of Asbt and Ost-alpha/beta may result in the intracellular accumulation of bile salts in cholangiocytes, which may play a role in the development of bile duct injury after liver transplantation. More clinical studies will be needed to study the changes in the expression of the bile salt transporters after liver transplantation in humans.

Although Asbt is only expressed in cholangiocytes, Ost alpha/beta is also expressed in hepatocytes. The observed changes in Ost-alpha/beta expression may, therefore, have resulted from changes in the expression in hepatocytes as well (25). However, we have no reasons to assume that expression of Ost alpha/beta is differently regulated in cholangiocytes and hepatocytes.
In conclusion, the current study indicates that Asbt expression is regulated in direct proportion to the biliary bile salt/phospholipid ratio. Although expression of Ost-alpha/beta normally follows the expression of Asbt, creating an effective cholehepatic shunt pathway, this balance may become disturbed after OLT, resulting in the intracellular accumulation of toxic bile salt in cholangiocytes. Intracellular accumulation of bile salts in cholangiocytes may aggravate biliary injury after OLT.

References


